Journal of Medical Virology 59:189–193 (1999)

Human Immunodeficiency Viruses Type 1 Subtypes Circulating in Spain

Africa Holguín,[1] Berta Rodés,[1] Ursula Dietrich,[2] and Vincent Soriano[1]

[1]*Service of Infectious Diseases, Instituto de Salud Carlos III, Madrid, Spain.*
[2]*Laboratory of Molecular Virology, Georg-Speyer-Haus, Frankfurt, Germany*

Genetic subtypes of Human immunodeficiency viruses type 1 (HIV-1) were investigated in 101 HIV-1-infected individuals living in Spain from 1993 to 1998. Samples selected randomly from the HIV clinic population included 29 Spanish native born subjects (28.7%) and 72 foreigners (71.3%). Proviral DNA extracted from peripheral blood mononuclear cells (PBMCs) or viral RNA isolated from plasma was amplified, and endonuclease restriction analysis was carried out on polymerase chain reaction (PCR) products. Restriction fragment length polymorphism (RFLP) analysis on the HIV-1 *protease* region enabled the characterisation of the different HIV genotypes infecting these individuals. Overall, 38 subjects (37.6%) carried non-B subtypes (A in 26, C in 2, D in 1, E in 2, and F in 7), 31 (81.6%) of them being immigrants. Direct sequence analysis of PCR products and/or a specific serological assay confirmed the data obtained by RFLP in most individuals tested. In conclusion, different HIV-1 subtypes are circulating currently in Spain, with non-B HIV-1 subtypes being confined mostly to immigrants. ***J. Med. Virol. 59:189–193, 1999.***

KEY WORDS: subtypes; HIV-1; HIV-2; RFLP; genotypes; viral load

INTRODUCTION

HIV shows a high degree of genetic variability in vivo [McCuthan et al., 1996; Hu et al., 1996; Coffin et al., 1995]. Analyses of HIV-1 gene sequences of virus strains from different geographical areas have revealed that HIV-1 can be divided into three distinct groups: M (major), O (outlier) [Myers et al., 1996; Peeters et al., 1997], and N (new) [Simon et al., 1998]. HIV-1 group M isolates can be subdivided further into at least ten distinct genetic subtypes, named from A to J. Subtype B predominates in North America and Europe [Hu et al., 1996; Myers et al., 1996, Janssens et al., 1997]. Subtypes are approximately equidistant from one to another in *env*. Two or more samples are required to define a new sequence subtype [Myers et al., 1996]. *Env* amino acid sequence variation within group M ranges from 3 to 23% amongst members of the same subtype and from 20 to 30% amongst members of different subtypes. The three groups, M, O, and N, differ by up to 35%. Subtype-dependent differences in frequency of usage of certain coreceptors for viral entry have been described [Tschering et al., 1998], although the syncytium-inducing phenotype has been noticed in all HIV-1 genetic subtypes [de Wolf et al., 1994; Nkengasong et al., 1995; Karita et al., 1997]. Thus, genetic subtypes may differ in important biological properties such as virulence, tissue tropism, and transmissibility. Moreover, differences in transcriptional regulation among HIV-1 subtypes could explain some of their differences in transmission and pathogenesis [Montano et al., 1997]. In this regard, a recent report indicated that HIV-1 subtype A, the most common variant found in some West African countries, causes progression of AIDS much more slowly than the rest of HIV-1 subtypes [Kanki et al., 1999].

There are good reasons for considering genetic subtyping as an important area in HIV/AIDS research. The different subtypes express distinct envelope proteins, which has important implications for the design and implementation of vaccine trials [Yue et al., 1994], since genetic variants can escape from the immune system [Arendrup et al., 1995]. Another problem resulting from genetic variation among subtypes is that some serological tests [Loussert-Ajaka et al., 1994], PCR diagnostic assays [Arnold et al., 1995; Brown et al., 1997], and viral load quantification methods [Dunne et al., 1997; Alaeus et al., 1997; Holguin et al., 1999] can fail to detect viruses belonging to some specific subtypes. Moreover, drug susceptibility could vary between different subtypes of HIV-1 [Palmer et al., 1998], although the phenotypic effect of nucleotide substitu-

Grant sponsor: Instituto de Salud Carlos III, Asociación Investigación y Educación en SIDA (AIES); and Comunidad Autónoma de Madrid (CAM).

*Correspondence to: Dr. Vincent Soriano, C/Rafael Calvo 7, 2° A, 28010-Madrid, Spain. E-mail vsoriano@dragonet.es

Accepted 30 March 1999

tions associated to drug resistance is still unclear for non-B isolates. All these limitations have important consequences at the clinical level. To understand better the HIV epidemic in the world, there is a need for data on the prevalence and geographic distribution of infections caused by viruses of a single subtype, as well as co-infections caused by two or more HIV-1 strains of distinct subtypes, which can subsequently be a source of recombination [Robertson et al., 1995].

Knowledge about the biological characteristics and the epidemic spread of the different HIV-1 subtypes is still poor. It has been very difficult to undertake large scale comparative studies of subtypes because of the lack of simple, rapid, and inexpensive tests for the recognition of HIV-1 subtypes. Serological subtyping can be used on a large scale at an affordable cost, but more research is needed to improve their specificity and sensitivity. HIV-1 serotyping is not sufficiently accurate to predict genetic subtypes [Hoelscher et al., 1998; Cheingsong-Popov et al., 1998]. Even different isolates can show discordances when comparing p24 antigen and *reverse transcriptase* activity [Corrigan et al., 1998]. Genetic techniques have also been developed allowing the classification of HIV-1 isolates into different genotypes. Although sequence analysis remains the most accurate genetic approach for the characterisation of viral genomes, other less expensive techniques based on polymerase chain reaction (PCR) have been used. The Heteroduplex Mobility Assay (HMA) has also been used for epidemiological studies [Delwart et al., 1993], allowing the screening of large sample sets in a short time. Efforts have been made to produce reference sets for standardisation of the HMA method [Bachmann et al., 1994].

MATERIALS AND METHODS

In order to investigate the distribution of different HIV-1 subtypes in Spain, a well-documented group of 101 HIV-1 seropositive individuals was examined. Twenty-nine specimens (28.7%) were selected randomly from HIV-1 Spaniards attended to between 1993 and 1998. The remaining 72 samples (71.3%) belonged to all HIV-1-positive foreigners who attended our clinic during the same period and for which specimens were available. Most of these immigrants were Africans, and nearly a third came from North Africa (Morocco and Algeria).

Restriction Fragment Length Polymorphism (RFLP) Analysis

Plasma HIV-RNA or proviral DNA from peripheral blood mononuclear cells (PBMCs) was purified, and sequential endonuclease restriction analysis was carried out on nested PCR products, according to conditions described previously [Janini et al., 1996]. The principle of the RFLP assay is based on the observed correlation between the restriction maps in specific viral regions and their phylogenetic classification, which is based on the sequence data [Myers et al., 1996; Janini et al., 1996]. Briefly, a 297 bp fragment of HIV-1 protease gene was amplified by PCR, and digested with *Alu1*. Cleavage fragments of 147 bp and 211 bp were obtained for subtypes B and D, while for subtypes A, C, and F a single 243 bp fragment was obtained. A sequential digestion with *Hinf1* on specimens harbouring the B/D restriction pattern enabled the differentiation between subtypes B and D, since only subtype B genomes has a restriction site for this endonuclease. For specimens showing the A/C/F restriction pattern after digestion with *Alu1,* a sequential digestion with *BclI* enabled the differentiation between subtype F and A/C, since only the former has a restriction site for this enzyme. Lastly, a digestion using *Scan1* enabled the differentiation between subtypes A and C. In summary, a sequential restriction site polymorphism within the HIV-1 *protease* gene of divergent HIV-1 strains results in the generation of diagnostic fragments of different lengths. Restriction patterns can be easily recognised by the different electrophoretic migration of fragments. In infections caused by single viral subtypes, a characteristic endonuclease restriction pattern is detected for phylogenetic subtypes A, B, C, D, and F, whereas in cases of multiple infections involving different HIV-1 subtypes, a mixture of restriction patterns is observed.

Genetic Sequence Analysis

For the HIV-1 *protease* gene, consensus nucleotide sequences (defined by the dominant base in the population of amplified HIV-1 genomes at each position) were determined by the *fmo1* method (Promega, Madison, WI) on nested PCR-amplified DNA. Sequence determinations were followed by treatment of the reaction mixture with terminal deoxynucleotidyltransferase to minimise ambiguities in sequencing gels derived from premature chain terminations. Sequence analysis of the HIV-1 V3 region was carried out in all available specimens from patients with non-B subtypes detected by RFLP. Near 600 bp of the *env* gene containing the V3 region were amplified by nested-PCR using combinations of 15 previously reported different primers [Yue et al., 1994; Delwart et al., 1993; Bachmann et al., 1994; Gehring et al., 1997] in several experiments. The nested PCR was done with an inner reverse biotinylated primer. For some non-B subtype specimens, repeated nested (inner) amplifications were needed to obtain sufficient genetic material to provide a visible band in the gel. The biotinylated PCR products were purified magnetically using dynebeads M280 (Dynal Inc., Fort Lee, NJ), according to the manufacturer's instructions. Direct sequencing of the PCR fragments was performed as previously described [Gehring et al., 1997]. Nucleotide sequences were aligned using the Clustal V program. The resultant alignment was edited by hand to align gaps to codon boundaries. A set of sequences representative of each of the HIV subtypes A through F was included in the analysis to identify the putative subtypes of the sequences obtained. Aligned sequences were used to generate a maximum-likelihood phylogenetic tree by Neighbour Joining and Distance Matrix methods.

TABLE I. Distribution of HIV-1 Subtypes in the Study Population According to RFLP Analysis

HIV-1 subtypes	Number	A	B	C	D	E	F
Spanish natives	29	3	23	1	1	1	1
Immigrants	72	23	40	1	0	1	6
Total	101	26	63	2	1	2	7

V3-Based Serotyping

Three peptides were used as antigens in a competitive ELISA. They were derived from an HIV-1 subtype B consensus, an Indian subtype C consensus, and a Thai subtype E consensus. Microplates were coated with the different peptides (one peptide per well). Serum samples were incubated in duplicates against all three peptides. Bound antibodies were detected by peroxidase-conjugated goat anti-human IgG using substrate OPD. The absorbance (OD) was measured at 492 nm. Values above the cut-off were graded from 1 to 4. The serotype of a serum was defined according to the seroreactivity pattern with all peptides [Gehring et al., 1997].

CD4+ Lymphocyte Count

The number of circulating CD4+ T cells was examined by flow cytometry (Coulter, Barcelona, Spain).

Viral Load Quantification

Plasma HIV-RNA was quantified using either a RT-PCR method (HIV Monitor, Roche, Spain) [Mulder et al., 1994] or the second generation branched-DNA assay (Quantiplex, Chiron, Madrid, Spain) [Kern et al., 1996], according to manufacturer's instructions.

RESULTS

Sixty-three subjects (62.4%) carried HIV-1 subtype B viruses, which was the most predominant subtype found in this study, either among Spanish native born subjects or immigrants (Table I). However, non-B subtypes were also recognized in 38 individuals, mainly among immigrants (81.6%). It is of interest that subtype B was recognized in 40 (55.6%) of 72 foreigners, but confined mainly to those from South America and North Africa, whereas all but one of subjects originating in Sub-saharan Africa carried non-B subtypes.

According to RFLP, subtype A was the most commonly found variant among non-B viruses (n = 26; 68.4% of them). Although three cases were recognised in Spanish born subjects, most carriers were immigrants, Liberia (n = 4), Nigeria (n = 4), and Equatorial Guinea (n = 5) being the most common places of birth.

Two subjects carried HIV-1 subtype C. One was a Spanish born male who had been in Kenya for long time and who most likely transmitted the infection to his spouse, an African woman. He admitted to have had multiple sexual contacts in Kenya before marriage. One subtype D was recognised in a Spanish born person. Unfortunately, information about the probable source of infection was not available. Two subtypes E were recorded in subjects from Zaire and Liberia, respectively. The first was a Spanish born woman who had spent several years in Zaire, where she acquired most likely the infection by heterosexual contact. Most individuals carrying HIV-1 subtype F were infected in African countries (Angola, Cameroon, Equatorial Guinea, and Senegal). Results are summarised in Table II.

Additional genetic sequence analyses in the *protease* and V3 regions, and specific serological analyses were performed in 29 subjects for which specimens were available. Six had been identified as belonging to subjects with subtype B, and the rest with non-B variants. The results confirmed the data obtained using RFLP genetic subtyping in most cases, although the sequencing of subtype F specimens are still in progress (data not shown).

It is interesting to note that plasma viral load was undetectable in many samples from patients carrying non-B subtypes (see Table II) despite their having low CD4 counts and being without antiretroviral therapy.

DISCUSSION

This study reports the first molecular characterisation of HIV-1 isolates from Spain using RFLP. The *protease*-based RFLP method reported previously [Janini et al., 1996] allowed us to recognise and distinguish infections caused by viruses of subtypes A, B, C, D, and F circulating in Spain. The two patients with subtype E viruses were detected using a specific serological assay [Gehring et al., 1997] in some previously noticed HIV-1 subtype A variants. As it is well known, subtypes A and E can not be distinguished by our protease-based RFLP analysis, because subtype E viruses are recombinants, *gag* and *pol* genes belonging to subtype A and the *envelope* gene to subtype E.

Our results emphasise that HIV-1 non-B subtypes are circulating currently in Spain. Although many of these have been observed among foreigners, some have been identified in native Spanish born persons. Among immigrants, Africans living in Spain are infected by a wide variety of HIV-1 genotypes and represent a potential source of introduction of HIV-1 non-B variants into the country, as previously noted by others in the US and Europe [Artenstein et al., 1995; Soriano et al., 1996; Lasky et al., 1997; Janssens et al., 1997].

The presence of non-B subtypes should be kept in mind for appropriate interpretation of serological and genetic diagnostic testing, as well as for the reliability of results obtained using current commercial viral load tests, which are based exclusively on B variants. These assays can misinterpret HIV-RNA values when testing specimens belonging to subjects infected with non-B variants [Arnold et al., 1995; Brown et al., 1997], as most likely occurred in many of our patients with low CD4 counts but undetectable plasma viraemia even in the absence of antiviral therapy. In a previous study [Holguín et al., 1999], each of the three different commercial methods for measuring plasma viraemia showed a different sensitivity when testing specimens from non-B carriers, ranging from 58% to 93%. The

TABLE II. Main Features of Individuals Infected With HIV-1 Non-B, Non-A Subtypes*

No.	Subtype	Country of birth	Suspected country of infection	Most likely route of infection	First diagnosis of HIV infection	Age (years)	Gender	CD4+ count ($\times 10^6$/l)	Plasma viraemia (HIV-1 RNA copies/ml)
1	C	Kenya	Kenya	Htsex	1995	29	F	370	<500
2	C	Spain	Kenya	Htsex	1995	47	M	160	23,040
3	D	Spain	Spain	Htsex	1994	29	F	NA	<500
4	E	Spain	Zaire	Htsex	1996	37	F	500	<500
5	E	Liberia	Liberia	Htsex	1997	31	F	NA	27,240
6	F	Portugal	Angola	Htsex	1997	NA	F	380	<500
7	F	Cameroon	Cameroon	Htsex	1997	29	F	48	784,300
8	F	Argentine	Argentine	IDU	1996	39	F	734	<500
9	F	The Netherlands	Overseas	Htsex	1990	66	M	228	26,320
10	F	Spain	Spain	Htsex	NA	NA	M	NA	NA
11	F	Equatorial Guinea	Equatorial Guinea	Htsex	1995	50	M	NA	617,940
12	F	Senegal	Senegal	Htsex	1991	30	M	264	<500

*F, female; M, male; NA, not available; Htsex, heterosexual; IDU, intravenous drug user.

RFLP method described above is useful genetic tool for assessing whether discordant CD4 counts and viral load values are in fact due to infections produced by non-B subtypes. Moreover, RFLP is a useful genetic tool for conducting surveillance by molecular epidemiology, allowing rapid recognition of infections caused by viruses of different HIV-1 subtypes.

ACKNOWLEDGMENTS

We thank M. Leal, A. Aguilera, A.M. Martin, A. Blesa, and E. Caballero for providing us some of the specimens tested in the study. We thank Dr. Danuta Pieniazek for providing support in the design and performance of the RFLP analysis. Lastly, we thank Mr. Peter Summerell for reviewing the manuscript.

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